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Clinical features of canine nodal T-cell lymphomas classified as CD8+ or CD4−CD8− by flow cytometry

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Abstract

Canine T-cell lymphoma (TCL) encompasses a heterogeneous group of diseases with variable clinical presentation, cytomorphology, immunophenotype, and biologic behaviour. The most common types of TCL in dogs involving peripheral lymph nodes include indolent T-zone lymphoma (TZL) and biologically aggressive peripheral T-cell lymphoma (PTCL). TCL phenotypes can be categorized by expression of the surface antigen molecules CD4 and CD8. The majority of TCL cases are $CD4^+$, with far fewer cases being $CD8^+$ or $CD4^-$ $CD8^-$. The clinical features of $CD4^+$ TCLs have been previously described. The less common TCL phenotypes, however, are poorly characterized with little to no information about prognosis. In this retrospective study, we describe and correlate the presenting clinical signs, flow cytometry, and outcomes of 119 dogs diagnosed with nodal, non-TZL, $CD8^+$ or $CD4^-$ $CD8^-$ TCL by flow cytometry. Skin lesions present at the time of diagnosis were more commonly observed in the $CD8^+$ TCL group. Mediastinal enlargement and/or hypercalcemia were more commonly seen in the $CD4^-$ $CD8^-$ TCL group. Dogs with either $CD8^+$ or $CD4^-$ $CD8^-$ TCLs had aggressive clinical disease with median overall survival (OS) times of 198 days and 145 days, respectively. In both groups, neoplastic cell size determined by flow cytometry ranged from small to large, and large cell size was associated with shorter OS times (median OS = 61 days). Cases classified as small cell had a median OS of 257 days. Expression levels of major histocompatibility complex (MHC) class II and CD5 were highly variable among cases but were not prognostically significant in this group of patients.

KEYWORDS

canine, CD4-CD8-, CD8 +, flow Cytometry, lymphoma, T-cell

1 | INTRODUCTION

Lymphoma is one of the most common malignancies in dogs, accounting for approximately 6% of all tumours and 90% of all haematopoietic neoplasms in this species.[1-5] T-cell lymphomas (TCLs) comprise approximately 30% of all lymphoma subtypes in dogs and encompass a heterogeneous group of diseases with variable clinical features and outcomes.[1,6-9] The most common subtypes of TCLs that involve one or more peripheral lymph nodes are T-zone lymphoma (TZL) and peripheral T-cell lymphoma (PTCL).[1,6] Distinction between these TCL subtypes is important due to differences in prognosis, with PTCL cases exhibiting significantly shorter survival times compared to TZL cases.[10,11]

TZL typically occurs in older patients and affected animals present with one or more enlarged lymph nodes and/or lymphocytosis.[10,12] Golden retrievers are overrepresented in the TZL patient

population in the United States, comprising nearly 50% of cases.[10,12] Unlike other types of TCL, mediastinal involvement and hypercalcemia are not typically seen with TZL.[10,11] TZL exhibits an indolent clinical course, with a median survival of 21.2 to 33.5 months.[10,11,13] TZL is diagnosed by a characteristic histomorphology and/or flow cytometry. Using flow cytometry, TZL is identified by a uniform expansion of small to intermediate-sized cells that express the T-cell antigens CD3 and CD5 with characteristic loss of the pan-leukocyte antigen CD45 and high expression of major histocompatibility complex (MHC) class II. TZL can express variable combinations of the T-cell subset antigens CD4 and CD8 including: $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$ or rarely $CD4^+CD8^+$.[10,14]

In contrast, PTCL affects middle-aged to older dogs and in addition to peripheral lymphadenopathy, dogs often present with concurrent hypercalcemia and/or cranial mediastinal enlargement.[11] PTCL can be readily distinguished from TZL by histology and flow cytometry.[1,6] By flow cytometry, PTCL is characterized by a uniform expansion of intermediate to large-sized cells that express the pan-leukocyte antigen CD45 and the T-cell antigens CD3 and/or CD5.[11] The majority of PTCL cases express the T-cell subset antigen CD4 and low levels of MHC class II.[11] The clinical and diagnostic features of $CD4^+$ PTCL have been previously described,[11] and it is considered an aggressive disease with median overall survival times of 159 to 162 days.[9,11,15] Boxer dogs are overrepresented in the $CD4^+$ PTCL population, comprising approximately 19% to 26% of cases.[11,16,17] In human patients, PTCL encompasses a heterogeneous group of TCLs, including PTCL-not otherwise specified (NOS), anaplastic large cell lymphoma (ALCL) and angioimmunoblastic lymphoma (AITL).[18,19] PTCL in canine patients appears to be most similar to PTCL-NOS in humans, while ALCL and AITL seem to be very rare in dogs.[6]

In a recent study, we evaluated paired flow cytometry and histology of lymph node biopsies in a large group of nodal, non-TZL ($CD45^+$), TCLs in dogs.[20] Similar to other reports, the majority of cases were classified as $CD4^+$ with fewer cases classified as $CD8^+$ and $CD4^-CD8^-$. Distinct histologic subtypes that correlated with T-cell subset antigen expression (CD4 and CD8) were not identified. All cases, including $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ TCLs, were classified as PTCL based on histologic appearance and the consensus diagnosis of three pathologists reviewing the histology independently. The significance of this finding was limited by the small number of $CD8^+$ (n = 5) and $CD4^-CD8^-$ (n = 5) cases included in the study and the intrinsic variability in cellular morphology within tumours classified as PTCL. Other studies focused on histologic and cytologic characterization of canine lymphomas using a variety of classification schemes other than the contemporary WHO standard, have similarly included small numbers of $CD8^+$ and/or $CD4^-CD8^-$TCLs. $CD8^+$ TCLs involving the lymph node have also been previously classified as mycosis fungoides, pleomorphic large cell, pleomorphic mixed cell, unclassifiable plasmacytoid, plasmacytoid, and unclassifiable high-grade.[21-23] $CD4^-CD8^-$ TCLs have been previously classified as lymphoblastic and PTCL.[21] The clinical features and biologic behaviour of these uncommon nodal $CD8^+$ and $CD4^-CD8^-$ TCL subtypes are poorly described and it remains uncertain if T-cell immunophenotype can be used to identify TCL subtypes with unique clinical presentations or survival times.

In the current study, we retrospectively evaluated a large cohort of canine patients diagnosed with nodal $CD8^+$ or $CD4^-CD8^-$ TCLs. We specifically aimed to characterize the presenting clinical signs, flow cytometry profile, and clinical outcomes associated with these rare TCL subtypes.

2 | METHODS

2.1 | Case selection

To determine the distribution of nodal lymphoma subtypes diagnosed at Colorado State University Clinical Immunology Laboratory (CSU-CI), the CSU-CI database was searched for canine lymph node aspirates submitted between 2012 and 2017. Cases with a final diagnosis of B-cell lymphoma, $CD4^+$ TCL, $CD8^+$ TCL, $CD4^-CD8^-$ TCL, $CD4^+CD8^+$ TCL or T-zone lymphoma were selected. Duplicate samples from the same patient with consistent diagnoses were eliminated, as were patients that were involved in studies that recruited dogs with specific lymphoma immunophenotypes.

Subsequently, cases with a diagnosis of either $CD8^+$ or $CD4^-CD8^-$ TCL were chosen for further evaluation. T-cell lymphoma cases were identified by a discrete population of T-cells with a single phenotype which comprised the majority of the sample, or a population of T-cells which exhibited at least one feature of aberrancy including: loss of both CD4 and CD8 T-cell subset antigens, loss of CD5 expression, marked decrease in MHC class II expression, or large cell size (greater than or equal to 1.3 times the size of normal T-lymphocytes). $CD8^+$ and $CD4^-CD8^-$ TCLs were characterized by CD3 and/or CD5 expression, lack of expression of CD21, and either $CD8^+CD4^-$ or $CD4^-CD8^-$ T-cell subset antigen expression patterns. Cases with morphology consistent with large granular lymphocytes (LGL-leukaemia) and cases with a diagnosis of T-zone lymphoma, identified by the characteristic loss of the pan-leukocyte marker CD45, were excluded from evaluation. Medical records for 120 of these cases were obtained for further evaluation. One case with a concurrent B-cell lymphoma was subsequently excluded from the case series, resulting in a total of 119 cases.

2.2 | Medical record evaluation

Medical records from patients that met inclusion criteria were acquired from the referring veterinarians. The patient age, sex, breed, date of diagnosis, type of treatment (categorized as: no treatment, corticosteroid treatment only, single-agent chemotherapy, or multi-agent chemotherapy), and date of last contact/euthanasia/death were extracted from the medical records. Patients in which the treatment regime or intention to treat was not clear in the medical record (n = 5) and patients who were enrolled in a novel immunotherapy clinical trial (n = 5) were removed from the group used to evaluate the effect of

treatment on overall survival. Patients who received a single injection of L-asparaginase and then were treated with CCNU alone or prednisone alone were categorized as single agent or corticosteroid alone, respectively. The presence or absence of the following clinical signs was also evaluated at the time of initial flow cytometry diagnosis: peripheral lymphadenopathy, abdominal lymphadenopathy, hepatomegaly, splenomegaly/splenic nodules, gastrointestinal involvement (including gastrointestinal mass, thickened intestines, and/or clinical diagnosis of protein-losing enteropathy), skin lesions, cranial mediastinal enlargement/sternal lymphadenopathy, and hypercalcemia. Where applicable, the diagnostic method used to identify these clinical abnormalities was also recorded. In cases where the presence or absence of the clinical sign was unknown, the patient was excluded from analysis of that particular feature. The patient's complete blood count (CBC) at the time closest to flow cytometry diagnosis was reviewed for hematologic abnormalities. Anaemia was classified as mild (haematocrit [HCT] 30%-36%), moderate (HCT 20%-30%), or marked (HCT <20%). Thrombocytopenia was characterized as mild (100 000-175 000 platelets/μL), moderate (30 000-100 000 platelets/μL), or marked (<30 000 platelets/μL) if there were no platelet clumps identified on blood smear review. Lymphocytosis was classified as mild (5000-10 000 lymphs/μL), moderate (10 000-30 000 lymphs/μL) or marked (>30 000 lymphs/μL).

2.3 | Flow cytometry

Flow cytometry was performed on lymph node aspirates in all cases using the methods described in Seelig et al[10] and the antibody panel described in Labadie et al.[24] The following criteria of the neoplastic population was evaluated in each case: cell size determined by median forward scatter of gated neoplastic cells, expression of the T-cell antigens CD3, CD5, CD4, and CD8 (classified as positive vs negative), and median fluorescence intensity (MFI) of MHC class II. T-cells from the lymph nodes of 13 normal dogs euthanized for IACUC-approved surgical continuing education courses were used as controls. The median cell size based on linear forward scatter and median MHC class II MFI of the $CD3^+CD5^+$ T-cells expressing either CD4 or CD8 from the control lymph nodes were measured and compared to the TCLs.

2.4 | Cytology

Of the 119 cases included in this study, only four cases (3 $CD4^-CD8^-$ TCL and 1 $CD8^+$ TCL) had cytology samples available at the CSU Veterinary Diagnostic Laboratory. To obtain additional cases for cytologic review, the CSU-CI database was searched for cases with concurrent flow cytometry and cytology where the slides were available at the CSU Veterinary Diagnostic Laboratory. Ten cases of $CD8^+$ TCL and 10 cases of $CD4^-CD8^-$ TCL were randomly selected for evaluation. Cases were classified as $CD8^+$ TCL and $CD4^-CD8^-$ TCL by flow cytometry using the same criteria described above. Fine needle aspirations from the 24 total cases were evaluated by two clinical pathologists (EDR, PRA). Pathologists were blinded to the immunophenotype. Additionally, for cytologic comparison, 10 $CD4^+$ nodal TCL samples with concurrent flow cytometry and cytology were randomly selected and reviewed.

2.5 | Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, San Diego, California) and R version 3.5.3. Fischer's exact test was used to compare categorical parameters and non-parametric Wilcoxon rank-sum test was used to compare continuous parameters between TCL phenotypes. Overall survival (OS) was calculated in days from the time of diagnosis by flow cytometry until time of death or censor by Kaplan-Meier method. Dogs still alive or lost to follow-up at the time of data analysis were censored at the last date of contact. The following potential clinical prognostic factors were evaluated: abdominal lymphadenopathy, sternal lymphadenopathy or mediastinal enlargement, cutaneous involvement, splenomegaly or splenic nodules, hepatomegaly, hypercalcemia, anaemia, age and treatment (multi-agent chemotherapy, single-agent chemotherapy, corticosteroids alone or no therapy). All clinical parameters were categorized as present or absent at the time of diagnosis. To evaluate age, patients were divided into “old” and “young” categories, with “old” defined as ≥10 years of age at the time of diagnosis. The following flow cytometry features of the neoplastic population were also evaluated: cell size as determined by forward scatter, MHC class II expression (MFI), and CD5 expression. Cases with a median neoplastic cell size greater than or equal to 1.3 times the median size of control T-cells from normal dogs were considered large. Cases with a median cell size less than 1.3 times the median size of control T-cells from normal dogs were considered small. MHC class II expression was categorized as high (greater than or equal to the median MFI of control T-cells from normal dogs) or low (less than the median MFI of control T-cell from normal dogs). Cases were categorized as CD5 positive or CD5 negative, as determined by complete loss of CD5 expression based on MFI and interpretation of the flow cytometry plots. The impact on survival was determined by log-rank test (*P*-value) and Cox proportional hazard model (Hazard Ratio [HR] and 95% confidence interval [CI]). Multivariable analysis was not performed due to insufficient sample numbers available for evaluation. A *P*-value less than .05 was considered significant.

3 | RESULTS

3.1 | Patient demographics

In dogs, $CD8^+$ and $CD4^-CD8^-$ TCLs involving the lymph node are rare (Figure 1). Between 2012 and 2017, a total of 11 746 unique cases of canine lymphoma involving the lymph node were diagnosed at the CSU-CI laboratory. Of this population, 8326 (70.9%) of cases were diagnosed as B-cell lymphoma, 1552 (13.2%) were diagnosed as $CD4^+$

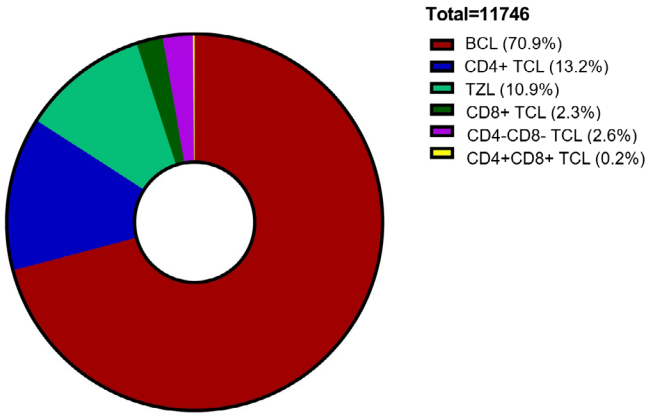

FIGURE 1 Immunophenotypic distribution of canine nodal lymphoid neoplasms. The immunophenotypic distribution of canine lymphoid neoplasia characterized by flow cytometry performed on lymph node aspirates between 2012 and 2017. B-cell lymphoma (BCL) is the most common immunophenotype, followed by $CD4^+$ T-cell lymphoma (TCL) and T-zone lymphoma (TZL). $CD8^+$ TCL, $CD4^-CD8^-$ TCL, and $CD4^+$ $CD8^+$ TCL are uncommon phenotypes

TCL, 1280 (10.9%) were diagnosed as T-zone lymphoma, 303 (2.6%) were diagnosed as $CD4^-CD8^-$ TCL, 267 (2.3%) were diagnosed as $CD8^+$ TCL, and 18 (0.2%) were diagnosed as $CD4^+CD8^+$ TCL. During this timeframe, a total of 561 cases met inclusion criteria for this study and medical records for 119 of these cases were obtained. Within this study group, 59 cases were categorized as $CD4^-CD8^-$ TCL and 60 cases were categorized as $CD8^+$ TCL. Patient demographics are summarized in Table 1. Significantly more patients diagnosed with $CD8^+$ TCL were categorized as older (≥10 years) than those diagnosed with $CD4^-CD8^-$ TCL (P = .0130). There was a significantly different sex distribution between $CD8^+$ TCL cases and $CD4^-CD8^-$ TCL cases (P = .0045), with $CD4^-CD8^-$ TCL having a male (62.7%) predominance and $CD8^+$ TCL having a female (63.3%) predominance. The most common breeds affected by $CD4^-CD8^-$ or $CD8^+$ TCLs were mixed breeds, Boxers, Golden Retrievers, and Labrador Retrievers. There was no difference in breed distribution between the two immunophenotypes (P = .8557).

3.2 | Clinical signs

Presenting clinical signs are summarized in Table 2. Most cases of $CD4^-CD8^-$ and $CD8^+$ TCLs in this study presented with peripheral lymphadenopathy, consistent with the inclusion criteria of lymph node sample. Abdominal lymphadenopathy was present in 81% of evaluated $CD4^-CD8^-$ TCLs and 56% of evaluated $CD8^+$ TCLs. Few cases demonstrated enlarged abdominal lymph nodes in the absence of reported peripheral lymph node involvement (1 $CD8^+$ TCL and 4 $CD4^-CD8^-$ TCLs). More commonly, dogs with peripheral lymphadenopathy had concurrent abdominal lymphadenopathy (17 $CD8^+$ TCLs and 25 $CD4^-CD8^-$ TCLs). Abdominal lymph node involvement was most often identified by abdominal ultrasound, with rare cases

TABLE 1 Summary of patient demographics

Immunophenotype	$CD4^-CD8^-$ TCL	$CD8^+$ TCL
Age, years (median; range)	8 (3-15)	10 (1-15)
Sex (number; percent)		
Female spayed	22 (37%)	36 (60%)
Female intact	0 (0%)	2 (3%)
Male castrated	32 (54%)	19 (32%)
Male intact	5 (8%)	3 (5%)
Breed (number; percent)		
Mixed breed	13 (22%)	12 (20%)
Golden retriever	10 (17%)	7 (12%)
Boxer	7 (12%)	7 (12%)
Labrador retriever	5 (8%)	8 (13%)
Other	24 (41%)	26 (43%)

Abbreviation: TCL, T-cell lymphoma.

identified by abdominal radiographs (n = 2). The presence of hepatomegaly was unknown in about half of the evaluated dogs and of those where is was evaluated, 50% (n = 18) of cases in the $CD8^+$ group and 47% (n = 16) of cases in the $CD4^-CD8^-$ TCL group were affected. Similarly, splenomegaly and/or splenic nodules affected 52% (n = 17) and 53% (n = 18) of evaluated cases in the $CD8^+$ and $CD4^-CD8^-$ TCL groups, respectively. Hepatomegaly and/or splenic changes were most often identified by abdominal ultrasound with fewer cases identified by abdominal radiographs (n = 6), computed tomography (n = 1), or abdominal palpation (n = 2). The cause of liver or splenic enlargement was not definitively determined by cytology or histology. Gastrointestinal involvement was uncommon among both TCL groups (n = 6) and included the presence of a gastrointestinal mass (n = 2), thickened intestines (n = 3), or clinical diagnosis of protein-losing enteropathy (n = 1).

Skin lesions were significantly more common in $CD8^+$ TCL patients, with 43% (n = 26) of the $CD8^+$ TCL cohort exhibiting skin lesions clinically suspicious for cutaneous lymphoma. Within the $CD8^+$ TCL group, 73% (n = 19) of the patients with skin lesions had mucocutaneous involvement. Thirteen of the 26 cases of $CD8^+$ TCL with cutaneous lesions were confirmed to be cutaneous lymphoma by cytology or histology. Nine of those cases were described specifically as epitheliotropic, one case was described as non-epitheliotropic, and epitheliotropism was unknown in the remaining cases. In contrast, only 10% (n = 6) of cases classified as $CD4^-CD8^-$ TCL presented with concurrent cutaneous involvement. Of these six total $CD4^-CD8^-$ TCL patients with cutaneous involvement, one dog had gingival involvement and the other five had generalized lesions. Two of the six cases within the $CD4^-CD8^-$ TCL group were confirmed to be cutaneous lymphoma by cytology or histology. Epitheliotropism was unknown in all the $CD4^-CD8^-$ TCL cases with cutaneous lesions. The presence of skin lesions was not significantly associated with cell size (P = .6785), expression of MHC class II (P = .6841), or CD5 expression (P = .8287) evaluated by flow cytometry.

TABLE 2 Summary of patient clinical signs and laboratory abnormalities at the time of presentation

	$CD4^{-}CD8^{-}$ nTCLs (n = 59)			CD8+ nTCLs (n = 60)			
Clinical signs	**% Affected**	**# Affected**	**# Evaluated**	**% Affected**	**# Affected**	**# Evaluated**	***P*-value***
Peripheral lymph nodes	93	55	59	98	59	60	.2068
Abdominal lymph nodes	81	29	36	56	18	32	.0380
Hepatomegaly	47	16	34	50	18	36	.8161
Splenomegaly	53	18	34	52	17	33	.9999
Gastrointestinal	14	4	28	8	2	26	.6702
Skin/mucocutaneous	10	6	59	43	26	60	<.0001
Sternal lymph nodes/mediastinum	60	21	35	13	5	40	<.0001
Hypercalcemia	29	16	55	4	2	54	.0005
Complete blood count							
Mild anaemia	11	5	44	10	4	41	.9999**
Moderate anaemia	14	6	44	15	6	41	
Severe anaemia	0	0	44	0	0	41	
Mild thrombocytopenia	27	12	44	7	3	41	.0323**
Mod. thrombocytopenia	11	5	44	10	4	41	
Severe thrombocytopenia	0	0	44	0	0	41	
Mild lymphocytosis	11	5	44	15	6	41	.4188**
Mod. lymphocytosis	2	1	44	2%	1	41	
Severe lymphocytosis	2	1	44	7%	3	41	

Abbreviation: TCL, T-cell lymphoma.
*Wilcoxon rank-sum test was performed to compare clinical features between $CD4^{-}CD8^{-}$ TCLs and $CD8^{+}$ TCLs.
**Complete blood count parameters were grouped to compare all cases with anaemia, thrombocytopenia, or lymphocytosis.

Cranial mediastinal enlargement and/or sternal lymphadenopathy was seen significantly more commonly in $CD4^{-}CD8^{-}$ TCLs, affecting 60% (n = 21) of evaluated cases. Mediastinal enlargement or sternal lymphadenopathy were diagnosed by thoracic radiographs in all cases, with no additional diagnostics performed. Similarly, hypercalcemia affected 29% (n = 16) of $CD4^{-}CD8^{-}$ TCL cases and was more frequently present at the time of diagnosis than in $CD8^{+}$ TCLs.

Complete blood counts (CBC) were available for review in 85 total cases (41 $CD8^{+}$ TCLs and 44 $CD4^{-}CD8^{-}$ TCLs) (Table 2). In general, CBC abnormalities were uncommon at the time of diagnosis. The most common changes included a mild to moderate anaemia, mild to moderate thrombocytopenia, and mild lymphocytosis (5000-10 000 lymphs/μL). Cases with mild to moderate neutrophilia in conjunction with mild to absent lymphopenia were common and interpreted as most consistent with a stress leukogram and not further evaluated. Two patients with $CD4^{-}CD8^{-}$ TCL were mildly neutropenic (1920 and 1940 neutrophils/μL) and this finding was not further explored. Moderate to marked lymphocytosis (>10 000 lymphs/μL), consistent with neoplastic involvement of the blood, was seen in three $CD8^{+}$ TCLs and one $CD4^{-}CD8^{-}$ TCL.

3.3 | Flow cytometry

All cases were evaluated for relative cell size based on median forward light scatter, MFI of MHC class II, and expression of CD5 (Figure 2, Table 3). There was no significant difference in cell size between $CD8^{+}$ TCLs and $CD4^{-}CD8^{-}$ TCLs (P = .3862), and neoplastic cell size ranged from small (less than 1.3 times the size of normal T-cells) to large (greater than or equal to 1.3 times the size of normal T-cells). The expression level of MHC class II was significantly lower in $CD4^{-}CD8^{-}$ TCLs, compared to $CD8^{+}$ TCLs (P = .0470). The number of $CD4^{-}CD8^{-}$ cases that were classified as low MHC expression (MFI less than normal T-cells), however, was not significantly different from $CD8^{+}$ cases (P = .0674). Loss of CD5 expression was observed significantly more often in $CD4^{-}CD8^{-}$ tumours compared to $CD8^{+}$ tumours (P = .0188).

3.4 | Cytology

Lymph node aspirates from 11 patients diagnosed with $CD8^{+}$ TCL by flow cytometry and 13 patients diagnosed with $CD4^{-}CD8^{-}$ TCL by flow cytometry were independently and blindly evaluated by two clinical pathologists (EDR, PRA) and compared to cytology from 10 cases of $CD4^{+}$ PTCL (representative examples in Figure 3). The 10 randomly-selected $CD4^{+}$ PTCL cases were morphologically similar and samples contained an expanded population of intermediate-sized lymphocytes with round to oval, occasionally indented nuclei, fine chromatin, absent to rarely one faint nucleolus, and moderate amounts of pale blue cytoplasm (Figure 3A). In comparison, $CD8^{+}$ and $CD4^{-}CD8^{-}$ TCLs were highly variable and there were no apparent trends in cytomorphology that correlated to immunophenotype. Approximately,

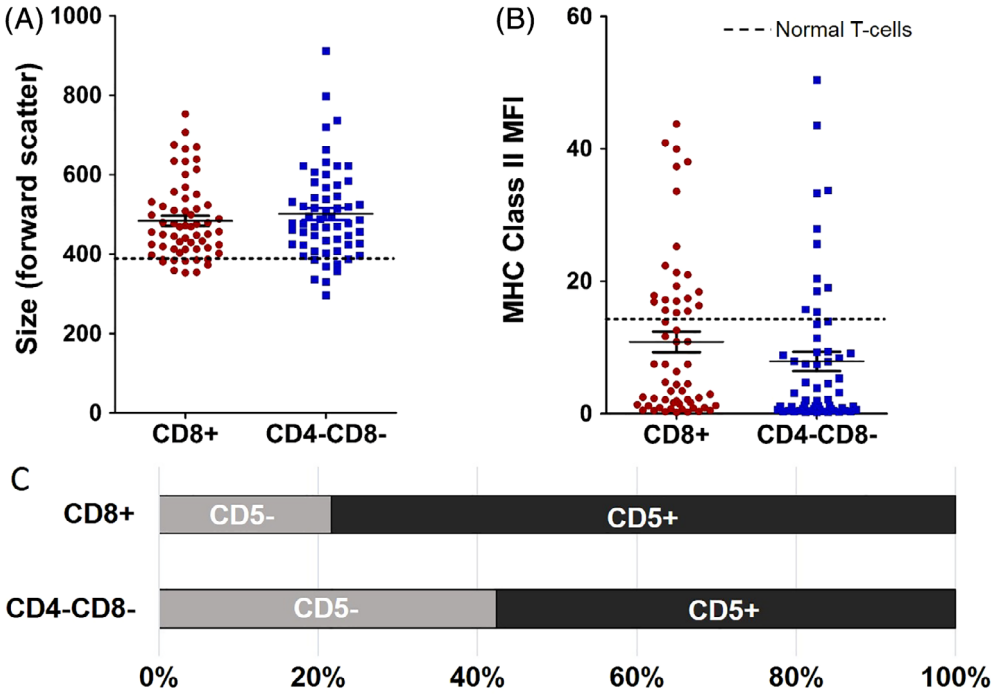

FIGURE 2 Flow cytometric features of $CD8^+$ and $CD4^-CD8^-$ TCLs. (A) The median neoplastic cell size as determined by forward scatter is depicted for each case. There is no significant difference in neoplastic cell size between $CD8^+$ and $CD4^-CD8^-$ TCLs. The median cell size of normal T-cells is represented by the dotted line. (B) The MHC class II median fluorescence intensity (MFI) on neoplastic T-cells is depicted for each case. There is variable expression of MHC class II expression in both $CD8^+$ and $CD4^-CD8^-$ TCLs, with some cases exhibiting very low expression and others with higher levels compared to normal T-cells (dotted line). (C) The percent of cases within each immunophenotypic group that have lost expression of CD5 is provided. Loss of CD5 expression was more commonly seen in $CD4^-CD8^-$ TCLs (*P*-value = .0188)

TABLE 3 Summary of flow cytometry features

	Size		MHC class II		Loss of CD5 expression	
Immunophenotype	Median	IQR	Median	IQR	Number	Percentage
$CD4^-CD8^-$ TCL	478	424-567	2.16	0.60-9.41	25	42
$CD8^+$TCL	463	412-529	5.60	1.40-17.16	13	22
Normal T-cells	387	373-412	13.78	9.81-18.52	0	0

Abbreviations: IQR, interquartile range; MHC, major histocompatibility complex; TCL, T-cell lymphoma.

half of the $CD8^+$ and $CD4^-CD8^-$ TCL cases had similar morphology to $CD4^+$ PTCL cases, but remaining cases were variable and often displayed more cellular pleomorphism. These cases tended to have round to indented nuclei and moderate amounts of cytoplasm similar to $CD4^+$ PTCL, but the nuclear chromatin was often coarser to irregularly clumped with more prominent nucleoli and the cytoplasm could become quite basophilic. Five cases had marked anisocytosis and anisokaryosis. In a small number of cases, neoplastic cells had one to few, small, clear, discrete cytoplasmic vacuoles. Four $CD8^+$ TCLs and 3 $CD4^-CD8^-$ TCLs had rare to small numbers of neoplastic lymphocytes with few fine azurophilic cytoplasmic granules.

3.5 | Clinical outcome

Of the 119 cases evaluated, nine cases died, 47 were humanely euthanized, and 63 cases were lost to follow up (median follow-up time: 64 days). All nine cases that died had progressive disease and were interpreted as a lymphoma-related death. Of the 47 cases that were humanely euthanized, 42 cases were euthanized due to progressive disease and declining quality of life. Four $CD8^+$ cases were euthanized with clinical signs of unconfirmed aetiologies including: haemoabdomen (n = 1), pleural effusion (n = 1), pulmonary nodules (n = 1), and renal failure (n = 1). A single $CD4^-CD8^-$ case was euthanized with renal failure.

$CD8^+$ TCLs and $CD4^-CD8^-$ TCLs were associated with poor clinical outcomes, with a median overall survival (OS) of 198 days (range 1-471 days) and 145 days (range 1-770 days), respectively (Figure 4A). Significant differences in survival between patients with tumours classified as $CD8^+$ TCLs vs $CD4^-CD8^-$ TCLs were not identified.

Log-rank and Cox proportional hazards univariable analyses were performed independently for both tumour subtypes and the results are summarized in Tables 4 and 5. For both subtypes, large cell size

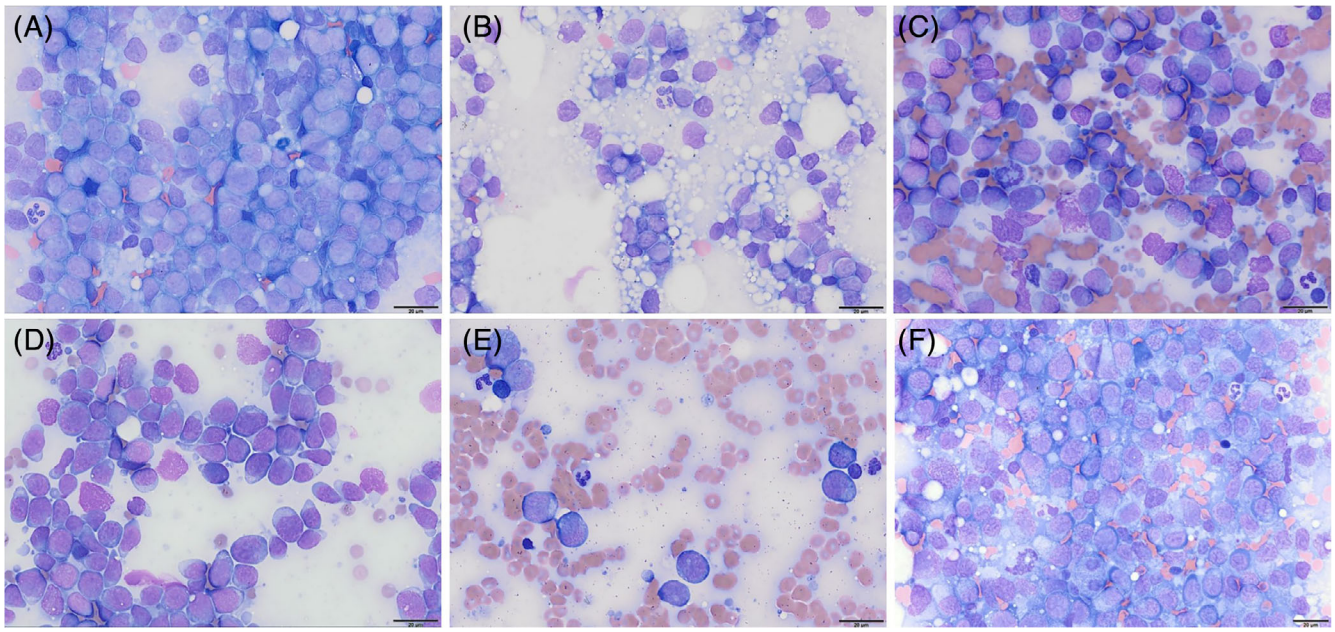

FIGURE 3 Cytomorphology of canine $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ T-cell lymphoma (TCL). Direct smears of fine-needle lymph node aspirates from a representative $CD4^+$ peripheral TCL case (A) and $CD8^+$ (B, D) and $CD4^-CD8^-$ (C, E, F) TCL cases with variable cytomorphology are shown; Wright Giemsa stain, ×50. (A) Ten $CD4^+$ peripheral TCLs were reviewed for comparison to $CD8^+$ and $CD4^-CD8^-$ TCLs and an example is shown here. The cells were intermediate sized with round to indented nuclei, fine chromatin, rare faint nucleoli and moderate amounts of pale blue cytoplasm. A subset of $CD8^+$ and $CD4^-CD8^-$ TCL cases had similar morphology to $CD4^+$ peripheral TCL (B), while remaining cases were more variable (C-F). These cases demonstrated more anisocytosis with some lymphocytes exceeding 20 μm in diameter, frequently coarser to irregularly clumped chromatin, and increased cytoplasmic basophilia. Small numbers of cells had small clear cytoplasmic vacuoles (D, F). One case had prominent nucleoli up to 8 μm in diameter (F)

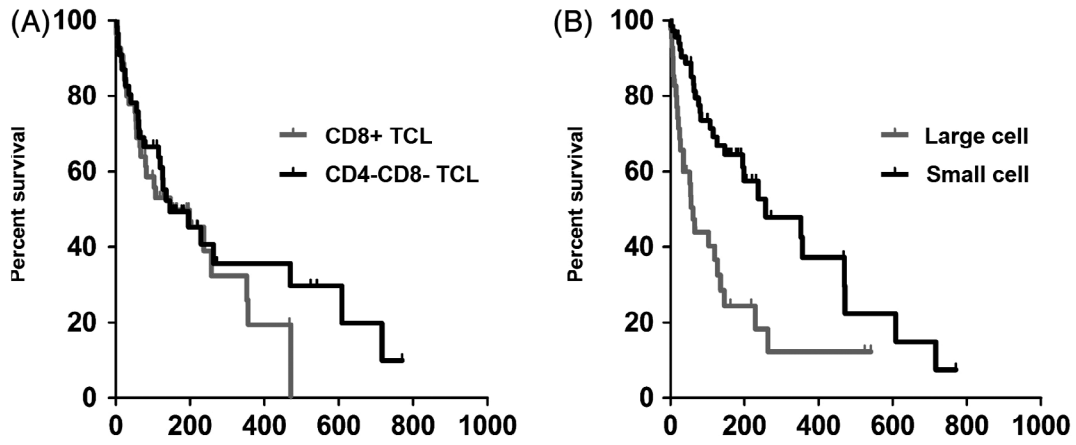

FIGURE 4 Overall survival of patients with $CD8^+$ and $CD4^-CD8^-$ T-cell lymphomas (TCLs). (A) Kaplan-Meier curves depicting overall survival from time of flow cytometry diagnosis for each immunophenotypic group are provided. There is no significant difference in overall survival between patients with $CD8^+$ and $CD4^-CD8^-$ TCLs. The median survival times were 198 days and 145 days for $CD8^+$ and $CD4^-CD8^-$ cases, respectively. (B) Neoplastic cell size measured by forward scatter by flow cytometry is associated with a shorter overall survival in patients with $CD8^+$ and $CD4^-CD8^-$ TCLs (P = .0002). The median survival time for cases classified as large cell was 61 days, while those classified as small cell was 257 days

(≥1.3 times normal T-cells) determined by forward scattering was associated with shorter overall survival. Combined, cases with large cell size had a median OS of 61 days (range 1-541 days) vs 257 days (range 1-770) for those with small cell size (P = .0002; Figure 4B). Neither MHC class II or CD5 expression levels had a significant impact on OS.

Among dogs with $CD8^+$ TCL, the presence of hepatomegaly, anaemia, or abdominal lymphadenopathy at the time of diagnosis

TABLE 4 Summary of survival analysis for selected clinical and immunophenotypic characteristics of $CD8^+$ TCLs

Variable		Condition	MST (d)	*P*-value	Hazard ratio (B/A)	CI, hazard ratio
Abdominal lymph nodes	A	Absent (n = 14)	198	.076	2.92	1.02-8.42
	B	Present (n = 18)	67			
Sternal lymph nodes/mediastinal mass	A	Absent (n = 35)	103	.717	1.80	0.23-14.39
	B	Present (n = 5)	Undefined			
Cutaneous involvement	A	Absent (n = 34)	80	.251	0.70	0.33-1.49
	B	Present (n = 26)	198			
Splenic changes	A	Absent (n = 16)	55	.351	0.86	0.26-2.89
	B	Present (n = 17)	107			
Hepatomegaly	A	Absent (n = 18)	257	.018	5.82	1.46–23.51
	B	Present (n = 18)	65			
Hypercalcemia	A	Absent (n = 52)	107	.376	Undefined	Undefined
	B	Present (n = 2)	Undefined			
Treatment: Chemo vs none	A	Multi/single (n = 45)	237	.002	6.92	2.09-22.87
	B	Steroids/none (n = 12)	19			
Treatment - Single vs multi	A	Multi-agent (n = 34)	237	.324	0.60	0.21-1.67
	B	Single-agent (n = 11)	257			
Anaemia	A	Absent (n = 31)	237	.001	6.64	2.13-20.69
	B	Present (n = 10)	54			
Age	A	Years >10 (n = 35)	198	.243	1.60	0.72-3.57
	B	Years <10 (n = 25)	80			
Cell Size	A	Small (n = 40)	237	.002	3.54	1.52-8.26
	B	Large (n = 20)	54			
CD5 Loss	A	CD5+ (n = 47)	147	.761	0.87	0.36-2.07
	B	CD5- (n = 13)	237			
Class II MHC	A	High (n = 30)	352	.562	0.80	0.37-1.72
	B	Low (n = 30)	147			

Abbreviations: CI, confidence interval; MHC, major histocompatibility complex; MST, median survival time; TCL, T-cell lymphoma.

were associated with a shorter OS. Patients with $CD8^+$ TCL and hepatomegaly at the time of diagnosis had a median OS of 65 days, while those without hepatomegaly had a median OS of 257 days (*P* = .018; HR 5.82, 95% CI 1.46-23.51). Patients with $CD8^+$ TCL who were anaemic at the time of diagnosis had a median OS of 54 days, while those patients without anaemia had median OS of 237 days (*P* = .001; HR 6.64, 95% CI 2.13-20.69). Patients with $CD8^+$ TCL and abdominal lymphadenopathy had a median OS of 67 days compared to 198 days in patients without abdominal lymphadenopathy (*P* = .076; HR 2.92 95% CI:1.02-8.42). Abdominal lymphadenopathy, hepatomegaly, or anaemia did not significantly impact survival in the $CD4^-CD8^-$ TCL group or the combined group of $CD8^+$ and $CD4^-CD8^-$ TCLs. The presence of cranial mediastinal enlargement/sternal lymphadenopathy, skin lesions, splenomegaly, hypercalcemia or age of the patient did not impact overall survival in $CD8^+$ and $CD4^-CD8^-$ TCLs.

Chemotherapeutic regimes used to treat the dogs in this retrospective study were widely variable in the combination of agents used, dosages, and frequency of administration. Consequently, to evaluate the impact of chemotherapy on survival, treatment protocols were assigned to the following broad categories: no treatment, corticosteroid therapy only, single-agent therapy, or multi-agent therapy. Corticosteroid therapy included treatment with prednisone, prednisolone, and/or dexamethasone only. Single-agent therapy included a single chemotherapeutic agent with or without concurrent corticosteroid therapy. Single agents used included CCNU and chlorambucil. Multi-agent therapy included two or more chemotherapeutic agents with or without corticosteroid therapy. Five patients were enrolled in a double-blinded clinical trial evaluating a monoclonal antibody and may have additionally received this novel therapeutic. All dogs enrolled in this immunotherapy trial (n = 5) and patients in which the therapeutic protocol was unclear in the medical record (n = 5) were removed from this outcome evaluation.

Within both TCL subgroups, dogs treated with chemotherapy (single-agent or multi-agent) had a significantly prolonged survival compared to those receiving no treatment or corticosteroids alone. Dogs with $CD8^+$ TCL treated with chemotherapy had an overall survival of 237 days compared to 19 days for those not receiving treatment or receiving corticosteroids alone (*P* = .002; HR 6.92, 95% CI:

TABLE 5 Summary of survival analysis for selected clinical and immunophenotypic characteristics of $CD4^{-}CD8^{-}$ TCLs

Variable		Condition	MST (d)	*P*-value	Hazard ratio (B/A)	CI, hazard ratio
Abdominal lymph nodes	A	Absent (n = 7)	145	.867	2.03	0.57-7.25
	B	Present (n = 29)	126			
Sternal lymph nodes/mediastinal mass	A	Absent (n = 14)	229	.179	0.38	0.13-1.11
	B	Present (n = 21)	469			
Cutaneous involvement	A	Absent (n = 23)	135	.769	1.51	0.51-4.44
	B	Present (n = 6)	115			
Splenic changes	A	Absent (n = 16)	145	.707	0.73	0.25-2.15
	B	Present (n = 18)	263			
Hepatomegaly	A	Absent (n = 18)	263	.648	1.05	0.36-3.01
	B	Present (n = 16)	195			
Hypercalcemia	A	Absent (n = 39)	145	.686	0.76	0.30-1.89
	B	Present (n = 16)	229			
Treatment: Chemo vs none	A	Multi/single (n = 40)	145	.050	3.09	1.00–9.54
	B	Steroids/none (12)	56			
Treatment: Multi vs single	A	Multi (n = 37)	195	.937	0.92	0.12-6.99
	B	Single (n = 3)	145			
Anaemia	A	Absent (n = 33)	469	.847	1.14	0.31-4.16
	B	Present (n = 10)	195			
Age	A	Old (n = 21)	126	.351	0.70	0.32-1.50
	B	Young (n = 38)	229			
Cell size	A	Small (n = 34)	469	.005	2.96	1.34-6.55
	B	Large (n = 25)	119			
CD5 loss	A	CD5+ (n = 34)	127	.351	1.43	0.67-3.04
	B	CD5– (n = 25)	469			
Class II MHC	A	High (n = 24)	126	.712	0.87	0.40-1.87
	B	Low (n = 35)	229			

Abbreviations: CI, confidence interval; MHC, major histocompatibility complex; MST, median survival time; TCL, T-cell lymphoma.

2.09-22.87). Similarly, dogs with $CD4^{-}CD8^{-}$ TCL treated with chemotherapy had an overall survival of 145 days compared to 56 days for those not receiving treatment or receiving corticosteroids alone (*P* = .050; HR 3.09, 95% CI: 1.00-9.54). No significant difference in overall survival was observed in patients treated with single-agent or multi-agent chemotherapy in either subgroup.

4 | DISCUSSION

$CD8^{+}$ and $CD4^{-}CD8^{-}$ TCLs involving the lymph nodes are rare in dogs, comprising 2.3% and 2.6% of all nodal lymphomas, respectively. The distribution of nodal lymphoma immunophenotypes in dogs from the CSU-CI database described in this study closely mimics the distribution reported by Martini et al, demonstrating inter-institutional consistency.[25] Because these lymphoma subtypes are uncommon, the clinical features and biologic behaviour has not previously been well described. In this retrospective study, we describe the clinical features of a large group of individuals diagnosed with $CD8^{+}$ and $CD4^{-}CD8^{-}$ TCLs by flow cytometry performed on lymph node aspirates. In general, patients with either $CD8^{+}$ or $CD4^{-}CD8^{-}$ TCL had poor clinical outcomes with median overall survival times of 198 and 145 days, respectively. There was no significant difference in survival between patients diagnosed with $CD8^{+}$ vs $CD4^{-}CD8^{-}$ TCLs and these survival times are similar to that previously reported for $CD4^{+}$ TCLs (159-162 days).[9,11,15]

$CD8^{+}$ and $CD4^{-}CD8^{-}$ TCLs had widely variable cytomorphology, with inconsistent cell size, chromatin pattern, cytoplasmic basophilia and presence of nucleoli. Similarly, by flow cytometry both $CD8^{+}$ and $CD4^{-}CD8^{-}$ neoplastic cells ranged in size from small to large. Cell size determined by flow cytometry did have a prognostic impact, with tumours comprised of large cells measuring greater than or equal to 1.3 times the median size of normal T-cells having a shorter overall survival. Nevertheless, the median overall survival of patients with small cell size was 257 days. This overall survival time is still much shorter than that expected for an indolent disease,[10,12] despite the small cell size. These results support the finding that cell size is prognostic only within specific subtypes of lymphoma,[11] but not as an indicator of indolent disease when the subtype is unknown. Therefore, these findings highlight the importance of ancillary diagnostics,

specifically flow cytometry, to phenotype canine lymphoma cases in order to provide accurate prognostic information and inform treatment decisions.

The retrospective nature of this study precluded complete WHO staging and sub-staging. Correlations between outcome and clinical presentation were limited by the lack of complete evaluation of bone marrow, tissue infiltrates, and peripheral blood involvement in the majority of cases. Further confirmation and characterization of neoplastic infiltrates within extranodal sites by cytology and flow cytometry could provide additional insights into potential differences between tumour subtypes and survival outcomes. Since these lymphoma subtypes are quite rare, accumulation of adequate numbers of cases to perform a prospective study with complete staging is challenging and was not attempted in the study.

Concurrent skin lesions, suspicious for or confirmed to be cutaneous lymphoma were more commonly seen in $CD8^+$ TCLs. This finding is consistent with previous reports demonstrating that canine cutaneous epitheliotropic lymphoma is most typically comprised of neoplastic $CD8^+$ T-cells.[25,26] Of the $CD8^+$ TCL cases with skin involvement, 73% had mucocutaneous localization. Cases with skin lesions did not exhibit distinct trends in cell size, expression of MHC class II, or CD5 expression evaluated by flow cytometry.

The presence of hepatomegaly, anaemia, or abdominal lymphadenopathy were associated with shorter overall survival times in cases of $CD8^+$ TCL. In dogs, hepatosplenic lymphoma is a rare form of T-cell lymphoma characterized by infiltration of the liver and/or spleen in the absence of peripheral lymph node involvement.[6,27] Hepatosplenic lymphoma in dogs can be comprised of $CD8^+$ or $CD4^-CD8^-$ T-cells.[27] Of the 18 $CD8^+$ TCLs in this study with hepatomegaly, one patient had abdominal lymphadenopathy in the absence of peripheral lymphadenopathy. All other $CD8^+$ TCLs and all cases of $CD4^-CD8^-$ TCL with hepatomegaly in this study had concurrent peripheral lymphadenopathy, making a diagnosis of hepatosplenic lymphoma less likely. Histopathology was not available for review in the examined cases. In a previous study, lymph node biopsies identified as $CD8^+$ and $CD4^-CD8^-$TCL by flow cytometry were given the histologic diagnosis of PTCL by a consensus diagnosis of three pathologists following independent review.[20] Based on the results of this previous report, we predict that most of the cases in this study would also be classified as PTCL by histology. A subset of cases in the current study were classified as small to intermediate in size by flow cytometry. This feature could be also be consistent with a classification of lymphoblastic lymphoma. Both PTCL and lymphoblastic lymphoma carry poor prognoses in dogs,[6] consistent with the overall survival times of patients evaluated in this study.

Presence of cranial mediastinal/sternal lymph node enlargement and/or hypercalcemia was more frequent in the $CD4^-CD8^-$ TCL subgroup. These clinical features have been previously associated with $CD4^+$ TCLs,[11] suggesting clinical similarity between these two entities. $CD4^-CD8^-$ TCLs also exhibit flow cytometry features characteristic of $CD4^+$ TCLs, including frequent low MHC class II expression and loss of CD5 expression.[11] Furthermore, a subset of $CD4^-CD8^-$ cases had cytomorphology similar to $CD4^+$ TCL. It is possible that at least a subset of $CD4^-CD8^-$ TCLs are part of the same, or closely related PTCL entity as $CD4^+$ PTCL. We hypothesize that $CD4^-CD8^-$ TCLs may arise from a thymic precursor prior to expression of CD4 and CD8, while $CD4^+$ TCLs arise from a slightly more mature thymocyte that has committed to the CD4 lineage. Alternatively, $CD4^-CD8^-$ TCLs may represent a variant of $CD4^+$ TCL, that has lost expression of CD4.

In this study, approximately 63% of dogs with $CD8^+$ TCL were female and approximately 63% of dogs with $CD4^-CD8^-$ TCLs were male. $CD4^+$ TCL in dogs also has a male predominance with 56% to 62% of affected patients being male.[11,20] In human patients, PTCL-NOS, which we hypothesize to be similar to canine $CD4^+$ TCL, has a reported male to female ratio of 2:1.[19] The pathobiology underlying this sex predilection is not yet understood.

Evaluation of the impact of treatment on overall survival is limited by the retrospective nature of this study. Within the patient group, a wide range in chemotherapy protocols were used with marked variations in drug combinations, dosages, and frequency of administration. Consequently, patients were divided into broad treatment categories (no treatment, corticosteroids only, single-agent, multi-agent) to evaluate the impact of treatment on survival. Additionally, complicating the treatment evaluation is inherent bias in treatment decisions based on disease severity at the time of presentation to the veterinarian, response to initial treatment, and/or financial and personal considerations of clients. With these limitations in mind, we did find that patients treated with single-agent or multi-agent chemotherapy protocols had a prolonged survival compared to those not receiving treatment or only receiving corticosteroid therapy. This finding was consistent in both $CD8^+$ and $CD4^-CD8^-$ TCL subgroups. In this study, a significant difference in survival was not observed between patients treated with multi-agent compared to single-agent chemotherapy regimens. The clinical significance of this finding is uncertain, given the limited power and sample size in this analysis. In this group of patients, CCNU was the most frequently administered single-agent therapeutic, and this drug has been shown to improve outcome in canine patients with cutaneous lymphoma.[28,29]

In human patients, PTCL is a heterogeneous disease with the majority of tumours classified into a "not-otherwise specified" subgroup (PTCL-NOS) of uncertain pathobiology.[30] Similar to dogs, PTCL-NOS in people is an aggressive disease with poor responses to conventional chemotherapy and 5-year overall survival rates of only 32%.[31] The majority of nodal PTCL-NOS in people are $CD4^+$, however cases can also be $CD8^+$, $CD4^-CD8^-$, or rarely $CD4^+CD8^+$.[32] In people, $CD8^+$ PTCL-NOS is more commonly associated with extranodal disease.[33] Similarly, extranodal T-cell lymphoma in dogs is most commonly $CD8^+$ or $CD4^-CD8^-$.[25] Gene expression profiling in human PTCL-NOS, has identified subgroups with expression profiles similar to mature, activated $CD4^+$ T-cells or less commonly, $CD8^+$ T-cells. In this human study, however, the tumours classified as $CD4^+$ "helper-like" or $CD8^+$ "cytotoxic-like" subgroups did not correlate with protein expression of CD4 or CD8 as evaluated by immunohistochemistry.[32] Consequently, the utility of classifying PTCL cases in humans based on surface expression of CD4 and CD8 molecules is uncertain.

In this study, we describe the clinical characteristics of two uncommon nodal TCL phenotypes in dogs. Due to the rarity of these TCL subtypes, previous reports of the clinical features and biologic behaviour are limited, preventing accurate prognostication of patients diagnosed with these tumours. We demonstrate that $CD8^+$ and $CD4^-CD8^-$ nodal TCLs have an aggressive behaviour with overall survival times similar to that of the more common $CD4^+$ PTCL. Furthermore, based on similar presenting clinical signs, cytomorphology and flow cytometry features, we suggest that at least a subset of $CD4^-CD8^-$ tumours may share a common pathogenesis to $CD4^+$ PTCL. $CD8^+$ TCLs exhibited more variable cytomorphology, as well as differences in clinical presentations and prognostic features. These findings imply that this entity may have a different pathophysiology to the more common $CD4^+$ TCL. Within both $CD8^+$ and $CD4^-CD8^-$ TCL groups, cell size was prognostic, and cases comprised of large cells determined by flow cytometry exhibited significantly decreased overall survival times. Importantly, however, even cases classified as small cell exhibited aggressive disease with shorter overall survival times than that reported for indolent lymphomas.[10,12,34,35] This study highlights the utility of flow cytometry in identifying and classifying prognostically-significant lymphoma subtypes.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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